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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 3041
Dale B. Schenk, et al.	Examiner: Kimberly A. Ballard
Application No.: 10/777,792	Art Unit: 1649
Filed: February 11, 2004	DECLARATION OF DALE B. SCHENK UNDER 37 C.F.R. § 1.131
For: PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE	
Customer No.: 20350	

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Commissioner for Patents
P.O. Box 1450
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Sir:

I, Dale Schenk, state as follows:

1. I understand the above-captioned application currently claims a composition comprising a fragment Aβ1-7 linked to a carrier which is a toxoid from a pathogenic bacterium to form a conjugate.

2. I am the inventor of the above claims.

3. During the period 1998-1999, I was Vice President, Neurobiology at Elan.

4. This declaration describes work performed in the United States of America by members of a research team at Elan under my direction.

5. Well before December 8, 1999, I had conceived of a composition comprising a fragment of Aβ1-5 linked to an immunoglobulin carrier and an adjuvant and successfully tested the fragment for its capacity to reduce amyloid deposits of Aβ in a transgenic

mouse model of Alzheimer's disease. I understand that the details of the experiment are reproduced in my patent application, US Application No. 09/201,430, filed November 30, 1998, at pp. 48-57 (Example IV), attached as Exhibit A. It can be seen from this Example that the experiment testing the Aβ1-5 conjugate was completed by at least November 30, 1998 (i.e., the filing date of the application).

6. Also attached as Exhibit B are copies of the notebook pages containing the data later incorporated into the US Application No. 09/201,430. The notebook pages are pp. 73-75 from notebook 2042 of my colleague Kelly Johnson-Wood. The dates on the pages have been redacted, but the work was necessarily completed before the filing of the patent application referred to in paragraph (5) on November 30, 1998. Page 73 summarizes the goals of the experiments ("to evaluate the Aβ and APP levels in the cortex, hippocampus and cerebellum of heterozygote PDAPP transgenic mice immunized with various forms/fragments of Aβ . . ." and also refers to the adjuvant as "CFA/IFA" (complete Freund's adjuvant/ incomplete Freund's adjuvant). Page 74 shows ELISA titers of Aβ in the cortex of PDAPP mice from immunization with the Aβ1-5 conjugate among other antigens and controls. Aβ1-5 was conjugated to sheep anti-mouse IgG via a cysteine link as discussed at p. 48, lines 28-32, of Exhibit A. Page 75 shows a scatter plot of the levels of Aβ in the brains of different PDAPP mice treated with the Aβ1-5 conjugate compared with controls and other immunogens. I concluded from these data that the Aβ1-5 conjugate was effective in reducing deposits of Aβ in the brain of a PDAPP transgenic mouse, which is a recognized animal model of Alzheimer's disease.

7. I had also conceived of a composition comprising an Aβ1-7 fragment linked to a carrier, and an adjuvant before December 8, 1999. This fragment was similarly tested in a PDAPP mouse model of Alzheimer's disease. I understand that the details of this experiment are reproduced in my patent application, US Application No. 09/580,015, filed May 26, 2000, at pp. 99-101 and 105-113 (Examples XVI and XIX), attached as Exhibit C.

8. Attached as Exhibit D is a memo between two of my colleagues, Nicky Vasquez and Don Walker that discusses synthesizing a peptide conjugate in which residues 1-7 of Aβ (DAEFRHD) are linked to a tetanus toxoid epitope carrier (peptide 1). The date of the memo has been redacted but it was well before December 8, 1999. This was the conjugate described in my U.S. Application No. 09/580,015.

9. Attached as Exhibit E is a document titled Chemical Registration for Non-Clinical Use compiled by my colleague, Don Walker. Elan scientists use these registrations to keep track of the structure of various compounds which are the subject of our research. This particular record indicates that ANKB5 has the amino acid sequence DAEFRHDQYIKANSKFIGITEL (i.e., the same sequence as peptide 1 in the memo discussed in paragraph (8)).

10. Attached as Exhibit F is notebook page 93 from notebook 2377 issued to my colleague M. Nguyen evidencing immunization with the peptide ANKB5, among other peptides, together with passive immunizations. The immunization was performed with complete Freund's adjuvant (CFA). The immunization with ANKB5 is the fourth typed paragraph from the top dated September 23 [1999]. The ANKB5 peptide is also referred to in the heading of this paragraph as "MAP."

11. Attached as Exhibit G is a copy of our records of immunizations and bleeds of PDAPP mice injected with ANKB5 aka MAP. It can be seen that the mice were injected and bled beginning September 23, 1999 as described in paragraph (10) and continuing with injections until the last injection was performed March 9 [2000].

12. After the last injection on March 9, 2000, the mice were sacrificed, brain tissue from the mice was extracted and homogenized, and Aβ levels were measured by ELISA assay, as generally described in Example XIX of Exhibit C (see particularly sections titled 4. Brain Tissue Preparation and 5. Measurement of Aβ levels at pp. 107-108). The data were then written up as a Quarterly Report dated April 20, 2000.

13. Attached as Exhibit H is a copy of the cover page and pp. 3, 4 and Figs. 10 and 11 from the Quarterly Report dated April 20, 2000. Pages 3-4 summarize the testing of the Aβ1-7 conjugate (also referred to as ANKB5 or MAP) among others. Fig. 10 shows a scatter plot and table of total Aβ levels in the cortex of PDAPP mice. Fig. 11 provides similar information for Aβ42 levels. The data shows that the Aβ1-7 conjugate reduced levels of total Aβ and Aβ42 in the cortex of the transgenic mouse model of Alzheimer's disease relative to control mice. I concluded from these data that the Aβ1-7 conjugate was effective in reducing levels of Aβ in the brain of the PDAPP transgenic mouse, which, as I have indicated above, is a recognized model of Alzheimer's disease.

14. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Dale B. Schenk

Date: Oct 29, 2007

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control animals did not respond to any of the Aβ proteins (Fig. 10, Lower Panel).

C. Conclusion

The results of this study show that AN1792 immunization of PDAPP mice possessing existing amyloid deposits slows and prevents progressive amyloid deposition and retard consequential neuropathological changes in the aged PDAPP mouse brain. Immunizations with AN1792 essentially halted amyloid developing in structures that would normally succumb to amyloidosis. Thus, administration of Aβ peptide has therapeutic benefit in the treatment of AD.

IV. Screen of Aβ Fragments

100 PDAPP mice age 9-11 months are immunized with 9 different regions of APP and Aβ to determine which epitopes convey the response. The 9 different immunogens and one control are injected i.p. as described above. The immunogens include four human Aβ peptide conjugates 1-12, 13-28, 32-42, 1-5, all coupled to sheep anti-mouse IgG via a cystine link; an APP polypeptide aa 592-695, aggregated human Aβ 1-40, and aggregated human Aβ 25-35, and aggregated rodent Aβ42. Aggregated Aβ42 and PBS are used as controls. Ten mice are used per treatment group. Titers are monitored as above and mice are euthanized at the end of 4 months of injections. Histochemistry, Aβ levels, and toxicology are determined post mortem.

A. Materials and Methods

1. Preparation of Immunogens

Preparation of coupled Aβ peptides: four human Aβ peptide conjugates (amino acid residues 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG) were prepared by coupling through an artificial cysteine added to the Aβ peptide using the crosslinking reagent sulfo-EMCS. The Aβ peptide derivatives were synthesized with the following final amino acid sequences. In each case, the location of the inserted cysteine residue is indicated by underlining. The

Aβ13-28 peptide derivative also had two glycine residues added prior to the carboxyl terminal cysteine as indicated.

Aβ1-12 peptide	NH_2-DAEFRHDSGYEVC COOH
Aβ1-5 peptide	NH_2-DAEFRC COOH
Aβ33-42 peptide	NH_2-C-amino-heptanoic acid-GLMVGGVVIA COOH
Aβ13-28 peptide	Ac-NH-HHQKLVFFAEDVGSNKGGC-COOH

To prepare for the coupling reaction, ten mg of sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) was dialyzed overnight against 10 mM sodium borate buffer, pH 8.5. The dialyzed antibody was then concentrated to a volume of 2 mL using an Amicon Centriprep tube. Ten mg sulfo-EMCS [N (ε-maleimidocuproyloxy) succinimide] (Molecular Sciences Co.) was dissolved in one mL deionized water. A 40-fold molar excess of sulfo-EMCS was added dropwise with stirring to the sheep anti-mouse IgG and then the solution was stirred for an additional ten min. The activated sheep anti-mouse IgG was purified and buffer exchanged by passage over a 10 mL gel filtration column (Pierce Presto Column, obtained from Pierce Chemicals) equilibrated with 0.1 M $NaPO_4$, 5 mM EDTA, pH 6.5. Antibody containing fractions, identified by absorbance at 280 nm, were pooled and diluted to a concentration of approximately 1 mg/mL, using 1.4 mg per OD as the extinction coefficient. A 40-fold molar excess of Aβ peptide was dissolved in 20 mL of 10 mM $NaPO_4$, pH 8.0, with the exception of the Aβ33-42 peptide for which 10 mg was first dissolved in 0.5 mL of DMSO and then diluted to 20 mL with the 10 mM $NaPO_4$ buffer. The peptide solutions were each added to 10 mL of activated sheep anti-mouse IgG and rocked at room temperature for 4 hr. The resulting conjugates were concentrated to a final volume of less than 10 mL using an Amicon Centriprep tube and then dialyzed against PBS to buffer exchange the buffer and remove free peptide. The conjugates were passed through 0.22 μ-pore size filters for sterilization and then aliquoted into fractions of 1 mg and stored frozen at -20°C. The concentrations of the conjugates were determined using the BCA protein assay (Pierce Chemicals) with horse IgG for the

standard curve. Conjugation was documented by the molecular weight increase of the conjugated peptides relative to that of the activated sheep anti-mouse IgG. The Aβ 1-5 sheep anti-mouse conjugate was a pool of two conjugations, the rest were from a single preparation.

2. Preparation of aggregated Aβ peptides

Human 1-40 (AN1528; California Peptides Inc., Lot ME0541), human 1-42 (AN1792; California Peptides Inc., Lots ME0339 and ME0439), human 25-35, and rodent 1-42 (California Peptides Inc., Lot ME0218) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. Of the four, AN1528 was the only peptide soluble at this step. A 100 µl aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspension was vortexed again and incubated overnight at 37°C for use the next day.

Preparation of the pBx6 protein: An expression plasmid encoding pBx6, a fusion protein consisting of the 100-amino acid bacteriophage MS-2 polymerase N-terminal leader sequence followed by amino acids 592-695 of APP (βAPP) was constructed as described by Oltersdorf et al., *J. Biol. Chem.* 265, 4492-4497 (1990). The plasmid was transfected into *E. coli* and the protein was expressed after induction of the promoter. The bacteria were lysed in 8M urea and pBx6 was partially purified by preparative SDS PAGE. Fractions containing pBx6 were identified by Western blot using a rabbit anti-pBx6 polyclonal antibody, pooled, concentrated using an Amicon Centriprep tube and dialysed against PBS. The purity of the preparation, estimated by Coomassie Blue stained SDS PAGE, was approximately 5 to 10%.

B. Results and Discussion

1. Study Design

One hundred male and female, nine- to eleven-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratory and Taconic Laboratory. The mice were sorted into ten groups to be immunized with different regions of Aβ or APP combined with Freund's adjuvant. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. The immunogens included four Aβ peptides derived from the human sequence, 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG; four aggregated Aβ peptides, human 1-40 (AN1528), human 1-42 (AN1792), human 25-35, and rodent 1-42; and a fusion polypeptide, designated as pBx6, containing APP amino acid residues 592-695. A tenth group was immunized with PBS combined with adjuvant as a control.

For each immunization, 100 μg of each Aβ peptide in 200 μl PBS or 200 μg of the APP derivative pBx6 in the same volume of PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 μl for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent four doses and with PBS for the final dose. Immunizations were delivered intraperitoneally on a biweekly schedule for the first three doses, then on a monthly schedule thereafter. Animals were bled four to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose.

2. Aβ and APP Levels in the Brain

Following about four months of immunization with the various Aβ peptides or the APP derivative, brains were removed from saline-perfused animals. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of Aβ and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein, the hemisphere was dissected and

homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and the level of amyloid or APP was quantitated by comparison to a series of dilutions of standards of Aβ peptide or APP of known concentrations in an ELISA format.

The median concentration of total Aβ for the control group immunized with PBS was 5.8-fold higher in the hippocampus than in the cortex (median of 24,318 ng/g hippocampal tissue compared to 4,221 ng/g for the cortex). The median level in the cerebellum of the control group (23.4 ng/g tissue) was about 1,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Woods et al., 1997, supra).

For the cortex, a subset of treatment groups had median total Aβ and Aβ1-42 levels which differed significantly from those of the control group ($p < 0.05$), those animals receiving AN1792, rodent Aβ1-42 or the Aβ1-5 peptide conjugate as shown in **Fig. 11**. The median levels of total Aβ were reduced by 75%, 79% and 61%, respectively, compared to the control for these treatment groups. There were no discernable correlations between Aβ-specific antibody titers and Aβ levels in the cortical region of the brain for any of the groups.

In the hippocampus, the median reduction of total Aβ associated with AN1792 treatment (46%, $p = 0.0543$) was not as great as that observed in the cortex (75%, $p = 0.0021$). However, the magnitude of the reduction was far greater in the hippocampus than in the cortex, a net reduction of 11,186 ng/g tissue in the hippocampus versus 3,171 ng/g tissue in the cortex. For groups of animals receiving rodent Aβ1-42 or Aβ1-5, the median total Aβ levels were reduced by 36% and 26%, respectively. However, given the small group sizes and the high variability of the amyloid peptide levels from animal to animal within both groups, these reductions were not significant. When the levels of Aβ1-42 were measured in the hippocampus, none of the treatment-induced reductions reached significance. Thus, due to the smaller Aβ burden in the cortex, changes in this region are a more sensitive indicator

of treatment effects. The changes in Aβ levels measured by ELISA in the cortex are similar, but not identical, to the results from the immunohistochemical analysis (see below).

Total Aβ was also measured in the cerebellum, a region typically unaffected in the AD pathology. None of the median Aβ concentrations of any of the groups immunized with the various Aβ peptides or the APP derivative differed from that of the control group in this region of the brain. This result suggests that non-pathological levels of Aβ are unaffected by treatment.

APP concentration was also determined by ELISA in the cortex and cerebellum from treated and control mice. Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α, the secreted form of APP which has been cleaved within the Aβ sequence), and full-length forms (FL) of APP, while the second recognizes only APP-α. In contrast to the treatment-associated diminution of Aβ in a subset of treatment groups, the levels of APP were unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with Aβ peptides are not depleting APP; rather the treatment effect is specific to Aβ.

In summary, total Aβ and Aβ1-42 levels were significantly reduced in the cortex by treatment with AN1792, rodent Aβ1-42 or Aβ1-5 conjugate. In the hippocampus, total Aβ was significantly reduced only by AN1792 treatment. No other treatment-associated changes in Aβ or APP levels in the hippocampal, cortical or cerebellar regions were significant.

2. Histochemical Analyses

Brains from a subset of six groups were prepared for immunohistochemical analysis, three groups immunized with the Aβ peptide conjugates Aβ1-5, Aβ1-12, and Aβ13-28; two groups immunized with the full length Aβ aggregates AN1792 and AN1528 and the PBS-treated control group. The results of image analyses of the amyloid burden in brain sections from these groups are shown in Fig. 12. There were significant reductions of amyloid burden in the cortical regions of three

of the treatment groups versus control animals. The greatest reduction of amyloid burden was observed in the group receiving AN1792 where the mean value was reduced by 97% ($p = 0.001$). Significant reductions were also observed for those animals treated with AN1528 (95%, $p = 0.005$) and the Aβ1-5 peptide conjugate (67%, $p = 0.02$).

The results obtained by quantitation of total Aβ or Aβ1-42 by ELISA and amyloid burden by image analysis differ to some extent. Treatment with AN1528 had a significant impact on the level of cortical amyloid burden when measured by quantitative image analysis but not on the concentration of total Aβ in the same region when measured by ELISA. The difference between these two results is likely to be due to the specificities of the assays. Image analysis measures only insoluble Aβ aggregated into plaques. In contrast, the ELISA measures all forms of Aβ, both soluble and insoluble, monomeric and aggregated. Since the disease pathology is thought to be associated with the insoluble plaque-associated form of Aβ, the image analysis technique may have more sensitivity to reveal treatment effects. However since the ELISA is a more rapid and easier assay, it is very useful for screening purposes. Moreover it may reveal that the treatment-associated reduction of Aβ is greater for plaque-associated than total Aβ.

To determine if the Aβ-specific antibodies elicited by immunization in the treated animals reacted with deposited brain amyloid, a subset of the sections from the treated animals and the control mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, Aβ-containing plaques were coated with endogenous IgG for animals immunized with the Aβ peptide conjugates Aβ1-5, Aβ1-12, and Aβ13-28; and the full length Aβ aggregates AN1792 and AN1528. Brains from animals immunized with the other Aβ peptides or the APP peptide pBx6 were not analyzed by this assay.

3. Measurement of Antibody Titers

Mice were bled four to seven days following each immunization starting after the second immunization, for a

total of five bleeds. Antibody titers were measured as Aβ1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with Aβ1-42. As shown in Fig. 13, peak antibody titers were elicited following the fourth dose for those four vaccines which elicited the highest titers of AN1792-specific antibodies: AN1792 (peak GMT: 94,647), AN1528 (peak GMT: 88,231), Aβ1-12 conjugate (peak GMT: 47,216)and rodent Aβ1-42 (peak GMT: 10,766). Titers for these groups declined somewhat following the fifth and sixth doses. For the remaining five immunogens, peak titers were reached following the fifth or the sixth dose and these were of much lower magnitude than those of the four highest titer groups: Aβ1-5 conjugate (peak GMT: 2,356), pBx6 (peak GMT: 1,986), Aβ13-28 conjugate (peak GMT: 1,183), Aβ33-42 conjugate (peak GMT: 658), Aβ25-35 (peak GMT: 125). Antibody titers were also measured against the homologous peptides using the same ELISA sandwich format for a subset of the immunogens, those groups immunized with Aβ1-5, Aβ13-28, Aβ25-35, Aβ33-42 or rodent Aβ1-42. These titers were about the same as those measured against Aβ1-42 except for the rodent Aβ1-42 immunogen in which case antibody titers against the homologous immunogen were about two-fold higher. The magnitude of the AN1792-specific antibody titer of individual animals or the mean values of treatment groups did not correlate with efficacy measured as the reduction of Aβ in the cortex.

4. Lymphoproliferative Responses

Aβ-dependent lymphoproliferation was measured using spleen cells harvested approximately one week following the final, sixth, immunization. Freshly harvested cells, 10^5 per well, were cultured for 5 days in the presence of Aβ1-40 at a concentration of 5 μM for stimulation. Cells from a subset of seven of the ten groups were also cultured in the presence of the reverse peptide, Aβ40-1. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Lymphocytes from a majority of the animals proliferated in response to PHA. There were no significant responses to the Aβ40-1 reverse peptide. Cells from animals immunized with the larger aggregated Aβ peptides, AN1792, rodent Aβ1-42 and AN1528 proliferated robustly when stimulated with Aβ1-40 with the highest cpm in the recipients of AN1792. One animal in each of the groups immunized with Aβ1-12 conjugate, Aβ13-28 conjugate and Aβ25-35 proliferated in response to Aβ1-40. The remaining groups receiving Aβ1-5 conjugate, Aβ33-42 conjugate pBx6 or PBS had no animals with an Aβ-stimulated response. These results are summarized in Table 5 below.

Table 5

Immunogen	Conjugate	Aβ Amino Acids	Responders
Aβ1-5	yes	5-mer	0/7
Aβ1-12	yes	12-mer	1/8
Aβ13-28	yes	16-mer	1/9
Aβ25-35		11-mer	1/9
Aβ33-42	yes	10-mer	0/10
Aβ1-40		40-mer	5/8
Aβ1-42		42-mer	9/9
r Aβ1-42		42-mer	8/8
pBx6			0/8
PBS		0-mer	0/8

These results show that AN1792 and AN1528 stimulate strong T cell responses, most likely of the $CD4^+$ phenotype. The absence of an Aβ-specific T cell response in animals immunized with Aβ1-5 is not surprising since peptide epitopes recognized by $CD4^+$ T cells are usually about 15 amino acids in length, although shorter peptides can sometimes function with less efficiency. Thus the majority of helper T cell epitopes for the four conjugate peptides are likely to reside in the IgG conjugate partner, not in the Aβ region. This hypothesis is supported by the very low incidence of proliferative responses for animals in each of these treatment groups. Since the Aβ1-5 conjugate was effective at significantly reducing the level

of Aβ in the brain, in the apparent absence of Aβ-specific T cells, the key effector immune response induced by immunization with this peptide appears to be antibody.

Lack of T-cell and low antibody response from fusion peptide pBx6, encompassing APP amino acids 592-695 including all of the Aβ residues may be due to the poor immunogenicity of this particular preparation. The poor immunogenicity of the Aβ25-35 aggregate is likely due to the peptide being too small to be likely to contain a good T cell epitope to help the induction of an antibody response. If this peptide were conjugated to a carrier protein, it would probably be more immunogenic.

V. Preparation of Polyclonal Antibodies for Passive Protection

20 non-transgenic mice are immunized with Aβ or other immunogen, optionally plus adjuvant, and are euthanized at 4-5 months. Blood is collected from immunized mice. Optionally, IgG is separated from other blood components. Antibody specific for the immunogen may be partially purified by affinity chromatography. An average of about 0.5-1 mg of immunogen-specific antibody is obtained per mouse, giving a total of 5-10 mg.

VI. Passive Immunization with Antibodies to Aβ

Groups of 7-9 month old PDAPP mice each are injected with 0.5 mg in PBS of polyclonal anti-Aβ or specific anti-Aβ monoclonals as shown below. All antibody preparations are purified to have low endotoxin levels. Monoclonals can be prepared against a fragment by injecting the fragment or longer form of Aβ into a mouse, preparing hybridomas and screening the hybridomas for an antibody that specifically

BBDC Study 006 — THERAPEUTIC EFFICACY OF Aβ FRAGMENTS

- to evaluate the Aβ and APP levels in the cortex, hippocampus, and cerebellum of heterozygote PDAPP transgenic mice immunized with various forms/fragments of Aβ as well as pBx6 (aa 590-625)

Immunogens: as noted in data tables
Adjuvant: CFA / IFA
Route of Administration: Intraperitoneally
Age at 1st Injection: ~ 9-10 months
Age at Termination: ~ 13-14 months

refer to K Barbours notebook regarding - Study 006 set-up and design → animal information / background, immunogen, immunogen preparation for injection, adjuvant, injection schedule, serum titers, ...

refer to Histology / Pharmacology notebooks / binders regarding termination procedures / protocols, perfusion, ...

refer to page 4, 2-5 of this notebook # 2042 for homogenization procedure and Aβ ELISA procedure (Total Aβ (266/3D6) and Aβ42 (21F12/3D6))
refer to pages 6-9 of this notebook # 2042 for APP ELISA procedure (APP-α/FL (8E5/2H3) and APP holoprotein (8E5/1C1))

data summarized and statistical analyses determined using StatView 4.51

EXHIBIT B

Continued on Page 74

Read and Understood By

Kathleen Wood — Signed — Date
John P Anderson — Signed — Date

Summary of total Aβ levels in cortex:

BBLOC STUDY 006 - THERAPEUTIC EFFICACY OF Aβ FRAGMENTS
Animal Types: 12 - 13 Month Old PDAPP Transgenic Mice
Assay: Total Aβ (266/3D6)
Termination Date: 060298, 060398, and 060598

CORTEX

PBS/ADJUVANT (CFA/IFA)		Aβ1-5		Aβ1-12		Aβ13-28		Aβ33-42	
Animal ID#	Aβ (ng/g)	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#
693 M	5584	1953	861* M	2400	843* M	1180	865* M	5710	845 M
859* M	2248	1840	803* M	4383	880 M	5905	828 M	5280	829 M
876* M	2858	980	2667-1 F	4273	620 F	11100	881* M	1468	864* M
856 M	3043	1380	2667-2 F	2275	637 F	288	805* F	7710	882* M
2658-1P F	8488	723	2591-1 F	4830	840 F	5487	806* F	1064	787* F
2658-3P F	11400	1998	2591-2 F	7405	841 F	6740	807* F	3880	758* F
2591-1P F	12750	7140	2591-3 F	6920	608 F	2880	836 F	350	758* F
2591-2P F	2120			16200	610 F	1333	837 F	8283	850 F
						6970	838 F	2116	851 F
								2837	852 F
Median	4221	1653	Median	4607	Median	5487	Median	3208	Median
		0.0109	p Value (M-W)	0.5285	p Value (M-W)	0.6304	p Value (M-W)	0.3284	p Value (M-W)
Mean	5884	2759	Mean	6086	Mean	4827	Mean	3838	Mean
St. Dev.	4293	2203	St. Dev.	4482	St. Dev.	3684	St. Dev.	2800	St. Dev.
% CV	76	80	% CV	74	% CV	76	% CV	73	% CV
	n = 8	n = 7		n = 8		n = 9		n = 10	
Mean Male	3163	1796	Mean Male	3392	Mean Male	6082	Mean Male	5041	Mean Male
St. Dev.	1638	203	St. Dev.	1402	St. Dev.	4982	St. Dev.	2608	St. Dev.
% CV	51	11	% CV	41	% CV	82	% CV	52	% CV
	n = 4	n = 2		n = 2		n = 3		n = 4	
Mean Female	8184	7444	Mean Female	7526	Mean Female	4210	Mean Female	3037	Mean Female
St. Dev.	4862	2689	St. Dev.	5258	St. Dev.	3240	St. Dev.	2846	St. Dev.
% CV	59	109	% CV	70	% CV	77	% CV	94	% CV
	n = 4	n = 5		n = 6		n = 6		n = 6	

CORTEX

pBx6 (aa590-895)		Aggregated Aβ25-35		Aggregated Aβ40		Aggregated Aβ42		Agg RODENT Aβ42	
Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#	Aβ (ng/g)	Animal ID#
8585	892 M	3290	806* M	3383	865* M	721	894 M	1480	848 M
4984	833 M	11500	884* M	2437	846 M	137	899 M	871	887* M
8617	849 M	6815	831 M	3898	2597 M	1330	860* M	870	891 M
8335	785 F	3410	898 M	954	2629-1 F	1050	878* M	117	897 M
1165	787* F	3260	2663-1 F	3460	2629-2 F	1259	2593B M	697	853 F
7150	783* F	2180	2663-2 F	41	2629-3 F	988	824 F	849	854 F
2080	765 F	4510	2594-1 F	3630	2629-4 F	952	827* F	1183	856 F
12300	2658-1A F	3067	2594-2 F	1557	2629-5 F	3768	828* F	3663	858 F
5810	2658-2A F	3257	2594-3 F			1520	829* F	1748	857 F
6617	Median	3290	Median	2900	Median	1050	Median	870	Median
0.6304	p Value (M-W)	0.7729	p Value (M-W)	0.1272	p Value (M-W)	0.0021	p Value (M-W)	0.0021	p Value (M-W)
6348.19	Mean	4388	Mean	2416	Mean	1301	Mean	1247	Mean
3392.11	St. Dev.	2904	St. Dev.	1422	St. Dev.	1007	St. Dev.	1030	St. Dev.
53.45	% CV	83	% CV	59	% CV	77	% CV	83	% CV
n = 8		n = 9		n = 9		n = 9		n = 9	
8721.89	Mean Male	6254	Mean Male	3232	Mean Male	887	Mean Male	760	Mean Male
1792.82	St. Dev.	3860	St. Dev.	739	St. Dev.	485	St. Dev.	571	St. Dev.
20.67	% CV	62	% CV	23	% CV	54	% CV	75	% CV
n = 2		n = 4		n = 3		n = 3		n = 4	
6138.33	Mean Female	3255	Mean Female	1926	Mean Female	1806	Mean Female	1637	Mean Female
4132.81	St. Dev.	832	St. Dev.	1570	St. Dev.	1333	St. Dev.	1207	St. Dev.
66.95	% CV	26	% CV	82	% CV	74	% CV	74	% CV
n = 6		n = 5		n = 5		n = 6		n = 6	

Aβ1-5, aggregated Aβ42 (human), and aggregated Aβ42 (rodent) treatments were efficacious as compared to PBS control for cortical total Aβ levels (Mann-Whitney → p value ≤ 0.05)

Continued on Page 75

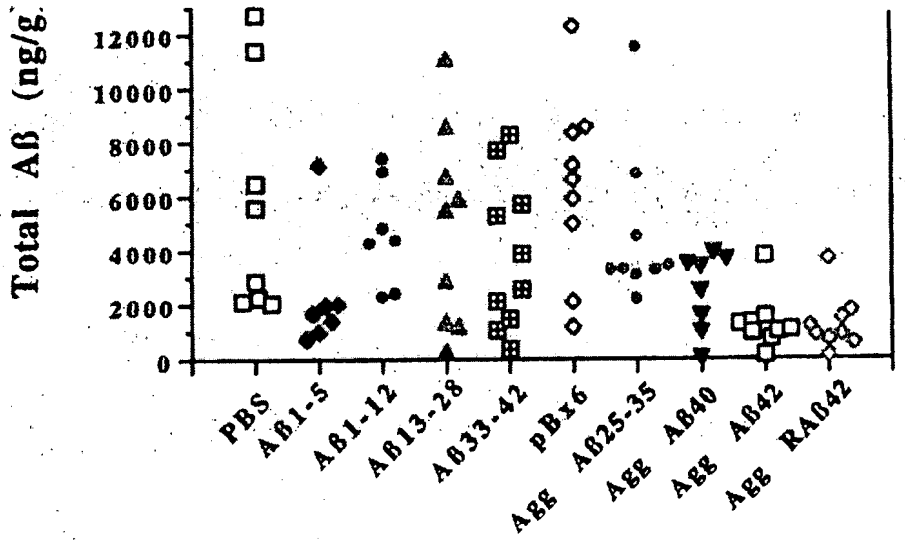
Read and Understood By

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PROJECT

Note Table: Attic 46 wild mouse cortex

	1	2	3	4	5	6	7	8	9	10
	PBS	Ab1-5	Ab1-12	Ab13-28	Ab33-42	pBx6	Agg Ab25-35	Agg Ab40	Agg Ab42	Agg Ab1RA042
1	6584.00	1652.50	2400.00	1160.00	9210.00	6584.00	3090.00	2367.50	721.40	1498.00
2	2547.50	1840.00	4383.33	9168.00	5280.00	4584.00	11500.00	2436.67	136.67	521.00
3	2858.00	979.50	4273.33	11102.00	1463.00	6416.67	6815.00	3898.00	1320.00	670.00
4	3047.50	1380.00	2775.00	268.00	7710.00	8335.00	3410.00	953.75	1050.00	117.00
5	6466.00	723.00	4810.00	5466.67	1264.00	1165.00	3260.00	3450.00	1259.00	697.00
6	11400.00	1937.50	7405.00	6740.00	3680.00	7150.00	2183.00	40.67	985.60	848.67
7	12750.00	7140.00	6820.00	2866.00	350.00	2090.00	4610.00	3630.00	961.83	1183.33
8	2120.00		16700.00	1332.50	8283.33	12300.00	3044.67	1556.67	3760.00	3443.33
9				8870.00	7115.00	5810.00	3256.67		1520.00	1745.00
10					2636.67					
11										



060908											
PBS											
	5370										
	2410										
	3300										
1690	1760										
	5730										
2110	1910										
Ab1-5											
1360	1820										
1780	2100										
949	1010										
1410	1350										
693											
2190	1910										
Ab1-12											
	2680										
	4350										
	3680										
	2320										
	5100										
Ab13-28											
1100	1260										
	5200										
	5420										
	5800										
	2860										
1410	1220										
8210	1860										
Ab33-42											
	5830										
	4930										
1260	1510										
998	1110										
	5450										
	1920										
	2390										
pBx6											
	5650			4770	4710	5290	4470	4984.00	490.69	9.85	833 M
	5670					7070	7110	6616.67	320.08	12.39	849 M
						8900	7770	8338.00	799.03	9.59	786 F
1260					1070			1165.00	134.35	11.53	767 F
						7150		7150.00	#DIV/0!	#DIV/0!	783 F
2210	1970							2090.00	169.71	8.12	765 F
						13200	11400	12300.00	1272.79	10.35	2658-1 F
	6230			6780	6660	4320	3760	5916.00	1219.38	20.64	2658-2 F
Agg Ab25-35											
	3290							3290.00	#DIV/0!	#DIV/0!	866 M
						11600	11400	11500.00	141.42	1.23	884 M
						7220	6410	6815.00	572.76	8.40	831 M
	3210					3610	3410	3410.00	200.00	5.87	856 M
	3390					3460	3130	3260.00	175.78	5.39	2663-1 F
2160	2200							2180.00	28.28	1.30	2663-2 F
	4190					5000	4340	4510.00	430.93	9.55	2594-1 F
	3410					3230	3360	3066.67	447.02	14.61	2594-2 F
	3460					1160	2950	3156.67	270.25	8.56	2594-3 F
Agg Ab40											
	3230		3730			3390	3050	3363.50	275.85	8.20	865 M
	2820		3240	2250				2436.67	332.01	13.63	846 M
	4040		4070	3540		4260	3580	3898.00	320.19	8.21	2597 M
893	877	1060	986					953.75	85.70	8.99	2629-1 F
	3590		3460			3300		3450.00	143.76	4.21	2629-2 F
				40.667				40.67	#DIV/0!	#DIV/0!	2629-3 F
	4000		3300			3950	3270	3630.00	369.08	10.99	2629-4 F
1240	1270	1620	1510	1940	1760			1556.67	234.57	12.64	2629-5 F
Agg Ab42											
699	745	805	705	667				721.40	58.95	8.18	694 M
		127	131	152				136.67	13.43	9.83	699 M
1130	1160	1510	1488					1328.00	202.81	15.26	860 M
1080	1060	1060	1000					1050.00	34.64	3.30	678 M
1280		1200	1140	1416				1259.00	119.35	9.48	25938 M
883		978	887		1010		1170	985.60	117.16	11.89	824 F
769		1170	1100	793	810	1070		951.83	180.34	18.95	627 F
	4110		3470	3810		4190	3260	3768.00	401.15	10.65	628 F
1210				1839				1529.00	438.41	28.84	629 F
Agg Rodent Ab42											
1380	1380							1680.00	141.42	9.56	848 M
571								571.00	#DIV/0!	#DIV/0!	867 M
861	879							870.00	12.73	1.46	691 M
				117				117.00	#DIV/0!	#DIV/0!	897 M
669	725							697.00	39.60	5.68	853 F
923					823		899	848.67	63.62	5.14	654 F
								1183.33	136.50	11.54	855 F
1060					1160	1330					
	3230					4090	3670	3663.33	430.04	11.74	856 F
1790	1700							1745.00	63.64	3.65	857 F

EXHIBIT B

Continued on Page 76

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decreased at day 1 and was absent by day 3. Thus, antibody-mediated phagocytosis of Aß leads to its degradation.

To determine if phagocytosis in the *ex vivo* assay was Fc-mediated, F(ab')2 fragments of the anti-Aß antibody 3D6 were prepared. Although the F(ab')2 fragments retained their full ability to react with plaques, they were unable to trigger phagocytosis by microglial cells. In addition, phagocytosis with the whole antibody could be blocked by a reagent against murine Fc receptors (anti-CD16/32). These data indicate that *in vivo* clearance of Aß occurs through Fc-receptor mediated phagocytosis.

XV: PASSAGE OF ANTIBODIES THROUGH BLOOD BRAIN BARRIER

This example determines the concentration of antibody delivered to the brain following intravenous injection into a peripheral tissue of either normal or PDAPP mice. PDAPP or control normal mice were perfused with 0.9% NaCl. Brain regions (hippocampus or cortex) were dissected and rapidly frozen. Brain were homogenized in 0.1% triton + protease inhibitors. Immunoglobulin was detected in the extracts by ELISA. Fab'2 Goat Anti-mouse IgG were coated onto an RIA plate as capture reagent. The serum or the brain extracts were incubated for 1hr. The isotypes were detected with anti-mouse IgG1-HRP or IgG2a-HRP or IgG2b-HRP (Caltag). Antibodies, regardless of isotype, were present in the CNS at a concentration that is 1:1000 that found in the blood. For example, when the concentration of IgG1 was three times that of IgG2a in the blood, it was three times IgG2a in the brain as well, both being present at 0.1% of their respective levels in the blood. This result was observed in both transgenic and nontransgenic mice - so the PDAPP does not have a uniquely leak blood brain barrier.

XVI: THERAPEUTIC EFFICACY OF AN Aß PEPTIDE IN MAP CONFIGURATION

A therapeutic adjuvant/immunogen efficacy study was performed in 9 - 10.5 month old male and female heterozygous PDAPP transgenic mice to test the efficacy of a fusion protein comprising Aß1-7 in tetrameric MAP configuration as described above. The duration of the study was 25 weeks with 29 – 40 animals per treatment group; therefore the animals were 15 – 16.5 months old at termination. The methodology used in this study is the same as that in the therapeutic study of different adjuvants in Example VIII above. . The treatment groups are identified in Table 18 below.

EXHIBIT C

Table 18

	Adjuvant	Immunogen	Dilution Buffer	Administration
Group 1:	CFA/IFA	MAP(Aß 1-7:TT) (100 µg)	PBS	IP (400 µl)
Group 2:	QS21	AN1792-GCS (75 µg)	PBS	SC (250 µl)
Group 3:	PBS	------	------	SC (250 µl)

Table abbreviations: MAP – multi-antigenic peptide; TT – tetanus toxoid t-cell epitope (830-844); SC – subcutaneous; IP – intraperitoneally; PBS – phosphate buffered saline; GCS is a glycine/citrate/sucrose formulation.

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The immunization schedule was identical for all of the treatment groups. The mice were injected on weeks 0, 2, 4, 8, 12, 16, 20, 24, with bleeds on week 3, 5, 9, 13, 17, 21 and 25. Groups 1, 2, 3, 4, and 6 received eight injections Groups 2 and 3, QS21/AN1792 and PBS respectively, served as the positive and negative controls for this study.

The titers were determined by the anti-Aß antibody titer assay.

Group 1, CFA/IFA:MAP(Aß1-7:TT) group, had low titer levels. The peak GMT reached was only 1,200 at 13 weeks, falling to a GMT of 600 by week 25. There were 3 of the 30 mice that did not raise any titer and another 7 mice that did not exceed a titer of 400 by the end of the study.

Group 2, the QS21/AN1792 control group, reached its peak titer at 17 weeks with a GMT of 16,000. The titer then fell over the next 8 weeks to finish with a GMT of 8,700. One animal in this group failed to raise a titer over the entire course of the experiment.

Group 3, the PBS alone group, had no titers.

Both treatment groups showed a significant lowing in cortical Aß levels as compared to the PBS control group (see Table 19). The CFA/IFA:MAP(Aß1-7) group, significantly lowered Aß as compared to the PBS control group in spite of the relatively low titers of anti-Aß antibodies.

Table 19 Cortical Aß levels

	PBS	MAP	QS-21
MEDIAN (ng/g tissue)	7,335	3,692	2,389
RANGE (ng/g tissue)	550 – 18,358	240 – 10,782	210 – 11,167
p value	----	0.0003	< 0.0001
N	38	30	34

In conclusion, the Aß 1-7MAP immunogen is effective in inducing a sufficient immune response significantly to retard Aß deposition in the cortex.

XVII. EPITOPE MAPPING OF IMMUNOGENIC RESPONSE TO Aβ IN MONKEYS

This example analyzes the response of a primate to immunization with AN1792 (i.e., Aβ1-42). Eleven groups of monkeys (4/sex/group) were immunized with AN1792 (75 or 300 μg/dose) in combination with QS-21 adjuvant (50 or 100 μg/dose) or 5% sterile dextrose in water (D5W, control group). All animals received IM injections on one of three injection schedules as shown in Table 20 for a total of 4, 5 or 8 doses. Serum samples (from 4 monkeys/sex/group) collected on Day 175 of the study and CSF samples (from 3 monkeys/sex/group) collected on Day 176 of the study (at the 6 month necropsy) were evaluated for their ability to bind to Aβ1-40 peptide and APP.

These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

Following baseline measures, patients begin receiving treatment. They are randomized and treated with either therapeutic agent or placebo in a blinded fashion. Patients are monitored at least every six months. Efficacy is determined by a significant reduction in progression of a treatment group relative to a placebo group.

A second phase II trial is performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI) or mild cognitive impairment (MCI), to probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease are selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population are collected. These patient populations are divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations are followed at intervals of about six months, and the endpoint for each patient is whether or not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

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XIX. General Materials and Methods

1. Measurement of Antibody Titers

Mice were bled by making a small nick in the tail vein and collecting about 200 µl of blood into a microfuge tube. Guinea pigs were bled by first shaving the back hock area and then using an 18 gauge needle to nick the metatarsal vein and collecting the blood into microfuge tubes. Blood was allowed to clot for one hr at room temperature (RT), vortexed, then centrifuged at 14,000 x g for 10 min to separate the clot from the serum. Serum was then transferred to a clean microfuge tube and stored at 4°C until titered.

Antibody titers were measured by ELISA. 96-well microtiter plates (Costar EIA plates) were coated with 100 µl of a solution containing either 10 µg/ml either Aβ42 or

SAPP or other antigens as noted in each of the individual reports in Well Coating Buffer (0.1 M sodium phosphate, pH 8.5, 0.1% sodium azide) and held overnight at RT. The wells were aspirated and sera were added to the wells starting at a 1/100 dilution in Specimen Diluent (0.014 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.6% bovine serum albumin, 0.05% thimerosal). Seven serial dilutions of the samples were made directly in the plates in three-fold steps to reach a final dilution of 1/218,700. The dilutions were incubated in the coated-plate wells for one hr at RT. The plates were then washed four times with PBS containing 0.05% Tween 20. The second antibody, a goat anti-mouse Ig conjugated to horseradish peroxidase (obtained from Boehringer Mannheim), was added to the wells as 100 µl of a 1/3000 dilution in Specimen Diluent and incubated for one hr at RT. Plates were again washed four times in PBS, Tween 20. To develop the chromogen, 100 µl of Slow TMB (3,3',5,5'-tetramethyl benzidine obtained from Pierce Chemicals) was added to each well and incubated for 15 min at RT. The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The color intensity was then read on a Molecular Devices Vmax at (450 nm - 650 nm).

Titers were defined as the reciprocal of the dilution of serum giving one half the maximum OD. Maximal OD was generally taken from an initial 1/100 dilution, except in cases with very high titers, in which case a higher initial dilution was necessary to establish the maximal OD. If the 50% point fell between two dilutions, a linear extrapolation was made to calculate the final titer. To calculate geometric mean antibody titers, titers less than 100 were arbitrarily assigned a titer value of 25.

2. Lymphocyte proliferation assay

Mice were anesthetized with isoflurane. Spleens were removed and rinsed twice with 5 ml PBS containing 10% heat-inactivated fetal bovine serum (PBS-FBS) and then homogenized in a 50° Centricon unit (Dako A/S, Denmark) in 1.5 ml PBS-FBS for 10 sec at 100 rpm in a Medimachine (Dako) followed by filtration through a 100 micron pore size nylon mesh. Splenocytes were washed once with 15 ml PBS-FBS, then pelleted by centrifugation at 200 x g for 5 min. Red blood cells were lysed by resuspending the pellet in 5 mL buffer containing 0.15 M NH4Cl, 1 M KHCO3, 0.1 M NaEDTA, pH 7.4 for five min at RT. Leukocytes were then washed as above. Freshly isolated spleen cells (10^5 cells per well) were cultured in triplicate sets in 96-well U-bottomed tissue culture-treated microtiter plates (Corning, Cambridge, MA) in RPMI 1640 medium (JRH Biosciences, Lenexa, KS)

supplemented with 2.05 mM L glutamine, 1% Penicillin/Streptomycin, and 10% heat-inactivated FBS, for 96 hr at 37°C. Various Aβ peptides, Aβ1-16, Aβ1-40, Aβ1-42 or Aβ40-1 reverse sequence protein were also added at doses ranging from 5 to 0.18 micromolar in four steps. Cells in control wells were cultured with Concanavalin A (Con A) (Sigma, cat. # C-5275, at 1 microgram/ml) without added protein. Cells were pulsed for the final 24 hr with 3H-thymidine (1 μCi/well obtained from Amersham Corp., Arlington Heights IL). Cells were then harvested onto UniFilter plates and counted in a Top Count Microplate Scintillation Counter (Packard Instruments, Downers Grove, IL). Results are expressed as counts per minute (cpm) of radioactivity incorporated into insoluble macromolecules.

4. Brain Tissue Preparation

After euthanasia, the brains were removed and one hemisphere was prepared for immunohistochemical analysis, while three brain regions (hippocampus, cortex and cerebellum) were dissected from the other hemisphere and used to measure the concentration of various Aβ proteins and APP forms using specific ELISAs (Johnson-Wood et al., supra).

Tissues destined for ELISAs were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0). The homogenates were mixed by gentle agitation using an Adams Nutator (Fisher) for three to four hr at RT, then stored at -20°C prior to quantitation of Aβ and APP. Previous experiments had shown that the analytes were stable under this storage condition, and that synthetic Aβ protein (Bachem) could be quantitatively recovered when spiked into homogenates of control brain tissue from mouse littermates (Johnson-Wood et al., supra).

5. Measurement of Aβ Levels

The brain homogenates were diluted 1:10 with ice cold Casein Diluent (0.25% casein, PBS, 0.05% sodium azide, 20 μg/ml aprotinin, 5 mM EDTA pH 8.0, 10 μg/ml leupeptin) and then centrifuged at 16,000 x g for 20 min at 4° C. The synthetic Aβ protein standards (1-42 amino acids) and the APP standards were prepared to include 0.5 M guanidine and 0.1% bovine serum albumin (BSA) in the final composition. The "total" Aβ sandwich ELISA utilizes monoclonal antibody monoclonal antibody 266, specific for amino acids 13-28 of Aβ (Seubert, et al.), as the capture antibody, and biotinylated monoclonal antibody 3D6, specific for amino acids 1-5 of Aβ (Johnson-Wood, et al), as the reporter

antibody. The 3D6 monoclonal antibody does not recognize secreted APP or full-length APP, but detects only Aβ species with an amino-terminal aspartic acid. This assay has a lower limit of sensitivity of ~50 ng/ml (11nM) and shows no cross-reactivity to the endogenous murine Aβ protein at concentrations up to 1 ng/ml (Johnson-Wood et al., supra).

The Aβ1-42 specific sandwich ELISA employs mAβ 21F12, specific for amino acids 33-42 of Aβ (Johnson-Wood, et al.), as the capture antibody. Biotinylated mAβ 3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of about 125 μg/ml (28 μM, Johnson-Wood et al.). For the Aβ ELISAs, 100 μl of either mAβ 266 (at 10 μg/ml) or mAβ 21F12 at (5 μg/ml) was coated into the wells of 96-well immunoassay plates (Costar) by overnight incubation at RT. The solution was removed by aspiration and the wells were blocked by the addition of 200 μl of 0.25% human serum albumin in PBS buffer for at least 1 hr at RT. Blocking solution was removed and the plates were stored desiccated at 4°C until used. The plates were rehydrated with Wash Buffer [Tris-buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5), plus 0.05% Tween 20] prior to use. The samples and standards were added in triplicate aliquots of 100 μl per well and then incubated overnight at 4° C. The plates were washed at least three times with Wash Buffer between each step of the assay. The biotinylated mAβ 3D6, diluted to 0.5 μg/ml in Casein Assay Buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added and incubated in the wells for 1 hr at RT. An avidin-horseradish peroxidase conjugate, (Avidin-HRP obtained from Vector, Burlingame, CA), diluted 1:4000 in Casein Assay Buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes at RT, after which the enzymatic reaction was stopped by the addition of 25 μl 2 N H2SO4. The reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

6. Measurement of APP Levels

Two different APP assays were utilized. The first, designated APP-α/FL, recognizes both APP-alpha (α) and full-length (FL) forms of APP. The second assay is specific for APP-α. The APP-α /FL assay recognizes secreted APP including the first 12 amino acids of Aβ. Since the reporter antibody (2H3) is not specific to the α-clip-site, occurring between amino acids 612-613 of APP695 (Esch et al., Science 248, 1122-1124 (1990)); this assay also recognizes full length APP (APP-FL). Preliminary experiments using

immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain homogenates of APP-FL suggest that approximately 30-40% of the APP-α /FL APP is FL (data not shown). The capture antibody for both the APP-α/FL and APP-α assays is mAb 8E5, raised against amino acids 444 to 592 of the APP695 form (Games et al., supra). The reporter mAb for the APP-α/FL assay is mAb 2H3, specific for amino acids 597-608 of APP695 (Johnson-Wood et al., supra) and the reporter antibody for the APP-α assay is a biotinylated derivative of mAb 16H9, raised to amino acids 605 to 611 of APP. The lower limit of sensitivity of the APP-αFL assay is about 11 ng/ml (150 ρM) (Johnson-Wood et al.) and that of the APP-α specific assay is 22 ng/ml (0.3 nM). For both APP assays, mAb 8E5 was coated onto the wells of 96-well EIA plates as described above for mAb 266. Purified, recombinant secreted APP-α was used as the reference standard for the APP-α assay and the APP-α/FL assay (Esch et al., supra). The brain homogenate samples in 5 M guanidine were diluted 1:10 in ELISA Specimen Diluent (0.014 M phosphate buffer, pH 7.4, 0.6% bovine serum albumin, 0.05% thimerosal, 0.5 M NaCl, 0.1% NP40). They were then diluted 1:4 in Specimen Diluent containing 0.5 M guanidine. Diluted homogenates were then centrifuged at 16,000 x g for 15 seconds at RT. The APP standards and samples were added to the plate in duplicate aliquots and incubated for 1.5 hr at RT. The biotinylated reporter antibody 2H3 or 16H9 was incubated with samples for 1 hr at RT. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbellipheryl-phosphate was added for a 30-min RT incubation and the plates were read on a Cytofluor tm 2350 fluorimeter (Millipore) at 365 nm excitation and 450 nm emission.

7. Immunohistochemistry

Brains were fixed for three days at 40C in 4% paraformaldehyde in PBS and then stored from one to seven days at 4°C in 1% paraformaldehyde, PBS until sectioned. Forty-micron-thick coronal sections were cut on a vibratome at RT and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in phosphate buffer) at -20°C prior to immunohistochemical processing. For each brain, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were incubated overnight with one of the following antibodies: (1) a biotinylated anti-Aβ (mAb, 3D6, specific for human Aβ) diluted to a concentration of 2 μg/ml in PBS and 1% horse serum; or (2) a biotinylated mAb specific for human APP, 8E5, diluted to a concentration of 3 μg/ml in PBS

and 1.0% horse serum; or (3) a mAb specific for glial fibrillary acidic protein (GFAP; Sigma Chemical Co.) diluted 1:500 with 0.25% Triton X-100 and 1% horse serum, in Tris-buffered saline, pH 7.4 (TBS); or (4) a mAb specific for CD11b, MAC-1 antigen, (Chemicon International) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (5) a mAb specific for MHC II antigen, (Pharmingen) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (6) a rat mAb specific for CD 43 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (7) a rat mAb specific for CD 45RA (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (8) a rat monoclonal Aβ specific for CD 45RB (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (9) a rat monoclonal Aβ specific for CD 45 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (10) a biotinylated polyclonal hamster Aβ specific for CD3e (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (11) a rat mAb specific for CD3 (Serotec) diluted 1:200 with 1% rabbit serum in PBS; or with (12) a solution of PBS lacking a primary antibody containing 1% normal horse serum.

Sections reacted with antibody solutions listed in 1,2 and 6-12 above were pretreated with 1.0% Triton X-100, 0.4% hydrogen peroxide in PBS for 20 min at RT to block endogenous peroxidase. They were next incubated overnight at 4°C with primary antibody. Sections reacted with 3D6 or 8E5 or CD3e mAbs were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.). Sections reacted with antibodies specific for CD 45RA, CD 45RB, CD 45, CD3 and the PBS solution devoid of primary antibody were incubated for 1 hour at RT with biotinylated anti-rat IgG (Vector) diluted 1:75 in PBS or biotinylated anti-mouse IgG (Vector) diluted 1:75 in PBS, respectively. Sections were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections were developed in 0.01% hydrogen peroxide, 0.05% 3,3'-diaminobenzidine (DAB) at RT. Sections destined for incubation with the GFAP-, MAC-1- AND MHC II-specific antibodies were pretreated with 0.6% hydrogen peroxide at RT to block endogenous peroxidase then incubated overnight with the primary antibody at 4°C. Sections reacted with the GFAP antibody were incubated for 1 hr at RT with biotinylated anti-mouse IgG made in horse (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:200 with TBS. The sections were next reacted for one hr with an avidin-biotin-peroxidase

complex (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:1000 with TBS. Sections incubated with the MAC-1-or MHC II-specific monoclonal antibody as the primary antibody were subsequently reacted for 1 hr at RT with biotinylated anti-rat IgG made in rabbit diluted 1:200 with TBS, followed by incubation for one hr with avidin-biotin-peroxidase complex diluted 1:1000 with TBS. Sections incubated with GFAP-, MAC-1- and MHC II-specific antibodies were then visualized by treatment at RT with 0.05% DAB, 0.01% hydrogen peroxide, 0.04% nickel chloride, TBS for 4 and 11 min, respectively.

Immunolabeled sections were mounted on glass slides (VWR, Superfrost slides), air dried overnight, dipped in Propar (Anatech) and overlaid with coverslips using Permount (Fisher) as the mounting medium.

To counterstain Aβ plaques, a subset of the GFAP-positive sections were mounted on Superfrost slides and incubated in aqueous 1% Thioflavin S (Sigma) for 7 min following immunohistochemical processing. Sections were then dehydrated and cleared in Propar, then overlaid with coverslips mounted with Permount.

8. Image Analysis

A Videometric 150 Image Analysis System (Oncor, Inc., Gaithersburg, MD) linked to a Nikon Microphot-FX microscope through a CCD video camera and a Sony Trinitron monitor was used for quantification of the immunoreactive slides. The image of the section was stored in a video buffer and a color-and saturation-based threshold was determined to select and calculate the total pixel area occupied by the immunolabeled structures. For each section, the hippocampus was manually outlined and the total pixel area occupied by the hippocampus was calculated. The percent amyloid burden was measured as: (the fraction of the hippocampal area containing Aβ deposits immunoreactive with mAb 3D6) x 100. Similarly, the percent neuritic burden was measured as: (the fraction of the hippocampal area containing dystrophic neurites reactive with monoclonal antibody 8E5) x100. The C-Imaging System (Compix, Inc., Cranberry Township, PA) operating the Simple 32 Software Application program was linked to a Nikon Microphot-FX microscope through an Optronics camera and used to quantitate the percentage of the retrospenial cortex occupied by GFAP-positive astrocytes and MAC-1-and MHC II-positive microglia. The image of the immunoreacted section was stored in a video buffer and a monochrome-based threshold was determined to select and calculate the total pixel area occupied by immunolabeled cells. For each section, the retrosplenial cortex (RSC) was manually outlined and the total pixel area

occupied by the RSC was calculated. The percent astrocytosis was defined as: (the fraction of RSC occupied by GFAP-reactive astrocytes) X 100. Similarly, percent microgliosis was defined as: (the fraction of the RSC occupied by MAC-1- or MHC II-reactive microglia) X 100. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240 μm intervals, were quantitated for each animal. In all cases, the treatment status of the animals was unknown to the observer.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.

From the foregoing it will be apparent that the invention provides for a number of uses. For example, the invention provides for the use of any of the antibodies to Aβ described above in the treatment, prophylaxis or diagnosis of amyloidogenic disease, or in the manufacture of a medicament or diagnostic composition for use in the same. Likewise, the invention provides for the use of any of the epitopic fragments of Aβ described above for the treatment or prophylaxis of amyloidogenic disease or in the manufacture of a medicament for use in the same.

05780.0005-00000

élan
pharmaceuticals
Memorandum

To: Don Walker

From: Nicki Vasquez

Date: [redacted]

Re: MAPs for Betabloc

Peptide synthesis for novel Betablock immunogens.

The goal of this project is to identify novel Betablock immunogens based on AN1792 B cell epitopes, and tetanus toxoid T cell epitopes. We will be comparing linear peptides to MAP4. Initially we would like to examine peptides containing single B cell epitopes, followed by peptides containing triplicate B cell epitopes.

Linear peptides (B cell epitope-T cell epitope):

1) DAEFRHD-QYIKANSKFIGITEL
(7) (15)

2) DAEFRHD-FNNFTVSFWLRVPKVSASHLE
(7) (21)

3) DAEFR-DAEFR-DAEFR-QYIKANSKFIGITEL
(15) (15)

4) DAEFR-DAEFR-DAEFR-FNNFTVSFWLRVPKVSASHLE
(15) (21)

The first phase of the project will be to look at the immune response to the linear peptides #1 and #2, followed by examination of the immune response to peptide #1-MAP4 and peptide #2-MAP4.

If the peptides #1 and #2 need to be prioritized, selection should be based on predicted ease of synthesis.

EXHIBIT D

021 SOP

Monday Sept. 20
PBS group Inject each mouse with 250 µl PBS subc

Tuesday Sept 21 MPL-SE
8.33 mls AN1792 + 2.5 mls of MPL-SE 1.2 mls PBS add sterile stir bar and mix while injecting. 250 µl SC

Wed Sept 22 ISA51
10 mls of Isa 51 in one 20 ml syringe. 8.33 mls AN1792+ 1.7 mls of PBS in the other 20 mls syringe(glass on order) attach with double luer lock barrone order) squeeze the oil into the liquid then back and forth 10 times. Erich Goldbach has done this before if you need help. 400 µl ip

Th. Sept 23 MAP

Weight out 5 mgs of ANKB5 (the first MAP peptide 019-1) Dissolve in 4.5 mls sterile di h2o plus 0.5 mls. 10 X PBS vortex. Add 5 mls of PBS and 10 mls of CFA sonicate and fill syringes. Inject 0.4 mls ip

Friday Sept. 24th
For the QS-21 and the QS-21 stop injection group: Injections one through 4

10 mgs of AN1792 using the non corrected mg/ml value of 0.6 mg/ml. 10/0.6=-16.6 mls of AN1792 plus 3 mgs (5 mls) QS-21 plus 3.4 mls of PBS. Add sterile stir bar so mixing can occur while injecting. Inject both groups with 250 µl of immunogen subc.

ISA

9/20/99
Inject passives
① 16611
② 1005
③ 306

Inject 021
① PBS

9/21/99
Inject passives → ① 27-1
② PBS
③ TM24

Inject 021 MPLse

Titer 018-2 passives & 016 repeats. Results shown on next page.

EXHIBIT F

Continued on Page

Read and Understood By

Signed 9/20/99 Date Signed 9/2/00 Date

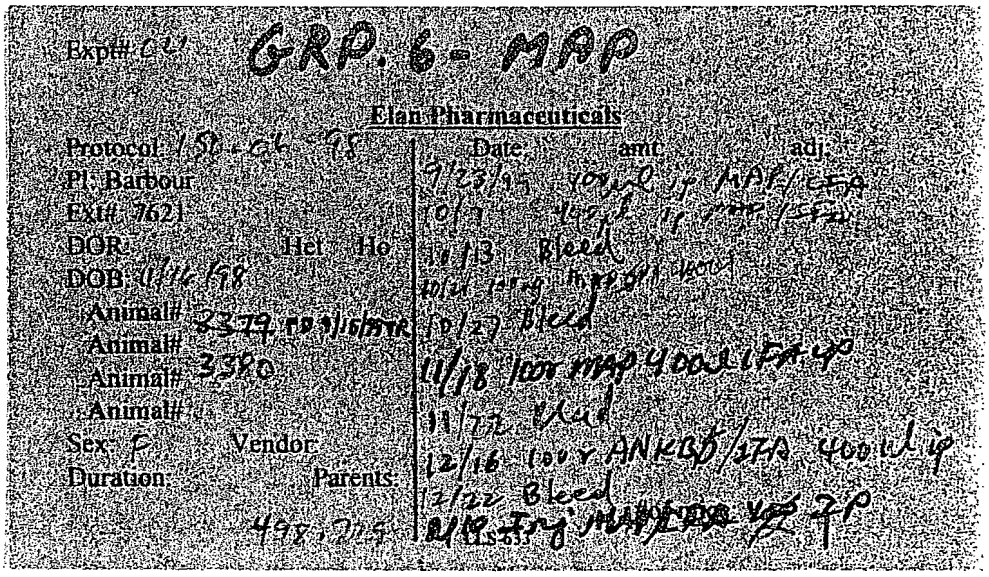
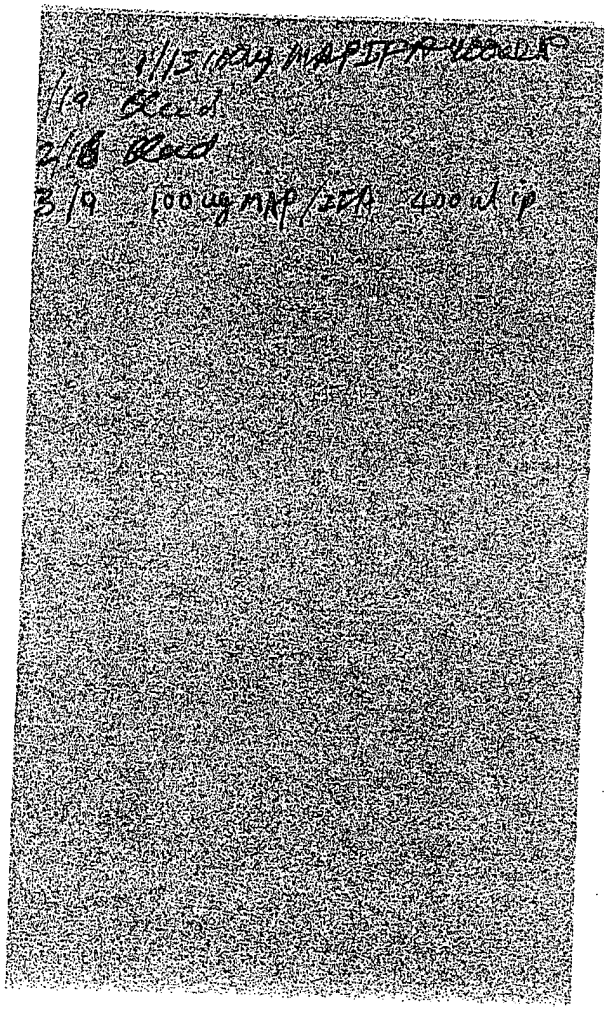

Expt# C21
GRP. 6 - MAP
Elan Pharmaceuticals
Protocol: 150-C6-98
PI: Barbour
Ext#: 7621
DOR: Het Ho
DOB: 11/16/98
Animal# 3379
Animal# 3380
Animal#
Animal#
Sex: F Vendor:
Duration: Parents:
498 / 775
Date amt adj
10/13 Bleed
10/27 Bleed
11/18 100 MAP 400 IFA 40
11/22 Bleed
12/16 100 ANKBP/IFA 400 IU 40
12/22 Bleed

EXHIBIT G


1/13 1824 MAP IP-Blood
1/19 Blood
2/18 Blood
3/19 100ug MAP/IFA 400 ul ip